# 流感疫苗接种前后尿液蛋白质组变化

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## 引言

尿液中的蛋白质是经过肾小球多次过滤血浆后富集起来的，其中的信息能较敏感的反映人体的变化。人们接种疫苗后，血液中抗体滴度以及阳转率检验一般在 2~4 周进行，那么尿液是否可以更早的观察到人体免疫反应的开始或者进程呢？接种同样的疫苗之后，不同人的自身免疫反应是否一致呢？我们招聘了 8 名志愿者（4 女，4 男）接种当年的四价流感病毒裂解疫苗。疫苗使用世界卫生组织（WHO）推荐的甲型和乙型流行性感冒病毒株，分别接种鸡胚，经培养、收获病毒液、灭活、浓缩、纯化、裂解后制成。该疫苗在进行临床试验后验证具有抗体保护，即接种本疫苗后，可刺激机体产生抗流感病毒的免疫力。我们发现每个人接种疫苗后的免疫反应不尽相同，尿液中和免疫反应有关的差异蛋白最早出现在接种后 24h 尿样中。

## 材料和方法

### 样品收集

8 名志愿者（4 女，4 男，21~29 岁）接种华兰生物疫苗有限公司的四价流感病毒裂解疫苗，在接种前留尿收集尿液，在接种后 24h 收集尿液；之后每隔 7 天收集一次尿液，共收集 4 次。该研究得到北京师范大学伦理委员会的批准，受试者均签署志愿者知情同意书。其中有三个时间点尿样，因为志愿者月经未能留下尿样或该时间点与其他事情冲突未能留下尿样。

蛋白质样品制备和胰蛋白酶酶解

尿液样品（n = 45），收集后储存在－80℃冰箱中，至全部收集结束。实验时，尿液样品取用 20 mL。使用 20 mmol / L 的二硫苏糖醇(DTT)在 37°C下与样品反应 1 h，使蛋白质结构中的二硫键变性，随后添加 55 mmol / L 的碘乙酰胺(IAA)并避光反应 30 min 以使二硫键结合位点烷基化。在-20°C下，用三倍体积的预冷酒精沉淀上清液 2 h，然后在 4 °C下，12,000 ×g 离心 30 min，获得蛋白沉淀。随后将沉淀物重悬于适量蛋白溶解液（8 mol/L 尿素，2 mol/L 硫脲，25 mmol/L DTT 和 50 mmol/L Tris）中。使用 Bradford 分析法测量蛋白质提取液浓度。通过使用滤器辅助样品制备（FASP）的方法，每个样品按 50：1 的比例使用胰蛋白酶（Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA）酶解 100 μg 的蛋白质。酶解后的多肽溶液使用 C18 Oasis HLB 柱（Waters, Milford, MA, USA）进行脱盐，然后通过真空离心浓缩仪进行干燥，干燥后的肽段密封至于-80 °C保存。

### 液相色谱-串联质谱（LC-MS / MS）分析

酶解后的肽段样品分别溶于 0.1%甲酸，每个样品取 1μg 肽段进行分析，液相为 Thermo EASY-nLC1200 色谱系统，连接预柱及分析柱。通过 Thermo Orbitrap Fusion Lumos 质谱系统(Thermo Fisher Scientific, Bremen, Germany) 采集数据。液相色谱分析方法：预柱：75 µm×2 cm, nanoViper C18, 2µm, 100Å；分析柱：50 µm×15 cm, nanoViper C18, 2µm, 100Å；进样体积：2 µL，流速：250 nL/min，流动相：A 相：100%质谱级水(Fisher Scientific,Spain)/1‰甲酸(Fisher Scientific)，B 相：80%乙腈(Fisher Scientific,USA)/20%水/1‰甲酸，120 min 梯度洗脱：0 min，3% B 相； 0 min-3 min，8% B 相；3 min- 93 min，22% B 相；93 min- 113 min，35% B 相；113 min- 120 min，90% B 相； 质谱分析方法，离子源：nanoESI，喷雾电压：2.2 kV，毛细管温度：320 °C，S-lens RF Level: 30，分辨率设置：一级（Orbitrap）120,000 @m/z 200，二级 30,000（Orbitrap）@m/z 200，母离子扫描范围：m/z 350-1350；子离子扫描范围：start from

m/z 110，MS1 AGC: 4e5，电荷范围：2-7，离子注入时间：50 ms，MS2 AGC: 1e5，离子注入时间：50ms，离子筛选窗口：2.0 m/z，碎裂模式：HCD，能量 NCE 32，Data-dependent MS/MS：Top 20，动态排除时间：15 s。将经胰蛋白酶酶解后浓缩干燥的样品复溶于 0.1％甲酸水溶液中。将 Thermo EASY-nLC 1200 色谱仪上加载预柱（75μm×2 cm, C18, 2μm, Thermo Fisher）和分析柱（50μm×15 cm, C18, 2μm, Thermo Fisher），流速为 250 nL / min。 A 相为 0.1％甲酸/水（Fisher Scientific, Spain），B 相为 80％乙腈（Fisher Chemical, USA）/0.1%甲酸/ 20％水。离子源为 nanoESI，由静电场轨道阱（Orbitrap）采集一级 MS 数据，分辨率为 120,000，电荷范围为 2-7，扫描质量范围为 m/z: 350-1,350，HCD 能量选择 32％。二级 MS 数据由 Orbitrap 收集，分辨率为 30,000。

**蛋白质鉴别和定量分析**

从液相-质谱获得的原始数据（RAW 文件），通过 Proteome Discoverer(version 2.1, Thermo Scientific) 和 MaxQuant (version 1.6.17.0)进行分析。通过 Xcalibur Qual Browser(version 3.0.63，Thermo Fisher Scientific)检查基峰总体情况。原始数据由 MaxQuant 使用大部分的默认参数处理。所有 RAW 文件在 MaxQuant 中同一个窗口进行处理，数据库搜索使用 Andromeda search engine（MaxQuant 中配置的），数据库采用 UniProt human sequence database (3 17, 2020; 196,111 sequences)。主搜索中前体质量容限设置在 4.5ppm，碎片质量容限设置在 20ppm。消化酶设置为 Trypsin/P 最大有两个错切位点。最小的肽段包括至少包括七个残基。蛋白质 N 端乙酰化和蛋氨酸的氧化作为可变修饰，半胱氨酸的脲甲基化（半胱氨酸碘乙酰化）作为固定修饰。由于还原烷基化以后，二硫键被打开，半胱氨酸会带上 carbamidomethyl 基团（+57Da），在搜库时，通常把 carbamidomethyl（C）选为固定修饰。对于没有修饰的肽段不设置 Andromeda 评分阈值，每一个鉴定到的修饰都有一个打分 Andromeda score 最小 40。肽段和蛋白的错误发现率设置为 1% 基于目标-诱饵反相数据库（target-decoy reverse database）。样本和样本之间每一个峰的洗脱时间尽量平行（Match between runs），match time window: 0.7 min, alignment time window: 20 min。选择无标记定量选项（LFQ），使用来自 MaxQuant 的 MaxLFQ 算法。

接下来使用 Perseus version (version 1.6.14.0)对数据进行预处理，去除污染和鉴别肽段数<1 的蛋白，取 LFQ intensity 的 $\log_2$ 进行归一化，我们认为缺失值是低于质谱检测的低丰度蛋白，不是随机缺失的（为了模拟低丰度 LFQ 值，用中值以下高斯分布中的随机值代替缺失值）缺失值以 0 替换。使用悟空平台进行非监督聚类分析（HCA），DAVID Bioinformatics Resources 6.8 进行生物学通路等分析。

## 结果

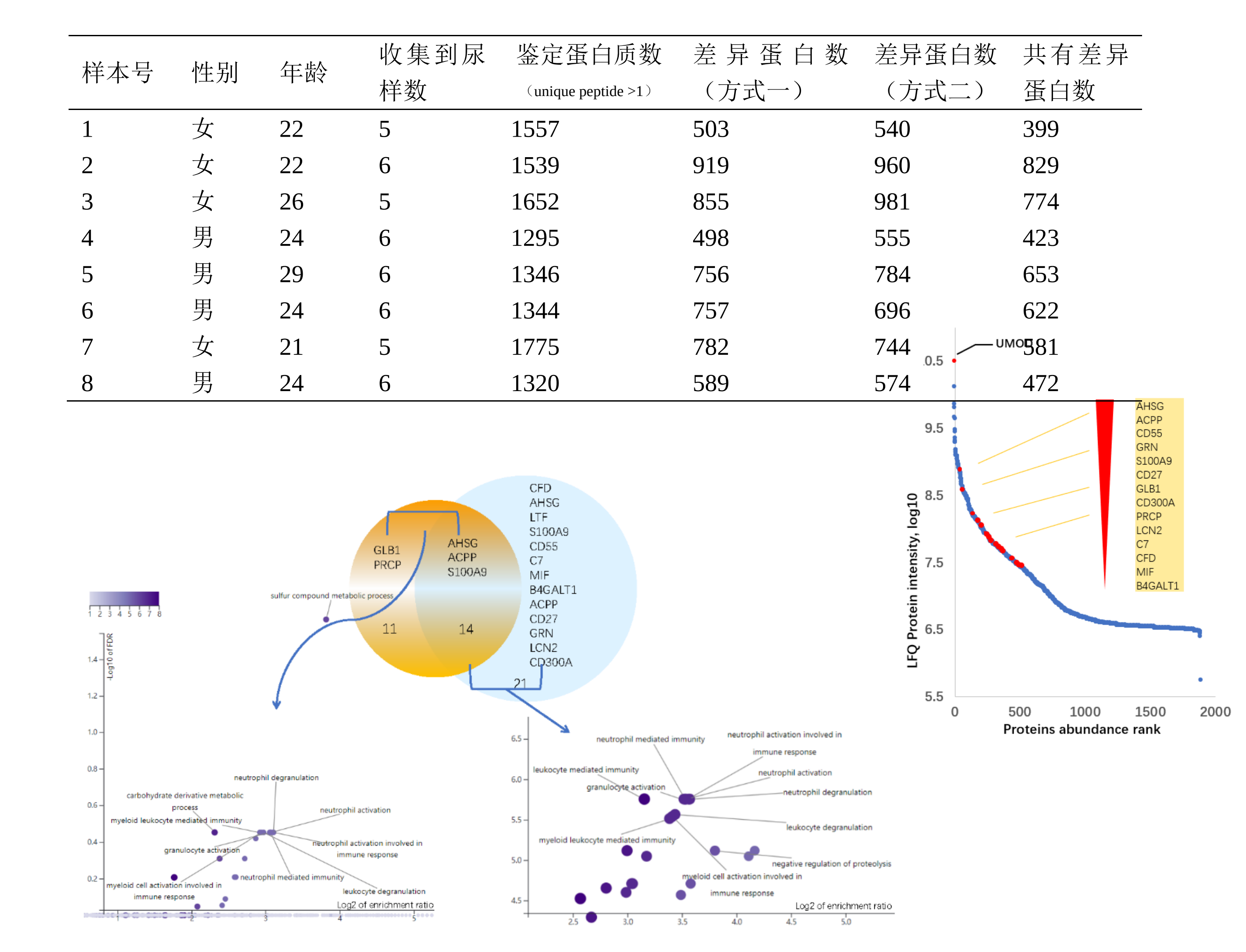
**通过 Bottom-up 蛋白质组学技术对尿样蛋白鉴别及差异蛋白分析**

对于每一个人的不同时间样本的尿样在 Maxquant 同一个窗口中归为一组进行鉴别分析，得到每个人的总蛋白总数接近。每一个时间点做三次技术重复，重复的数据中蛋白数和强度的拟合呈高斯分布且相似，可信度较高。总共有 32415 胰蛋白酶酶切的肽段被鉴定到，包含蛋白质 N 端乙酰化和蛋氨酸的氧化的肽段。这些肽段归属于 5199 不同的的蛋白组（proteins group），每组蛋白中有平均接近 10 个的肽段数被鉴定到，鉴定序列覆盖度为 30.9%，其中 Q 值<1%且特异肽段数>1 的高可信度蛋白有 1893 个，尿液蛋白质组跨度超过 6 个数量级，表明了良好的覆盖深度，如图。其中每个人的自身情况和鉴定到的蛋白数，如表。

每个人在接种流感疫苗之前收样一次，接种后 24h，7 天，14 天，21 天，28 天均收样，我们通过两种比较方式找寻差异蛋白，如附图示意，方式一是以接种前为对照组，其后 5 次分别与其比较得到差异蛋白；方式二是邻近的两次收样进行比较得到差异蛋白，差异蛋白筛选条件均为 $p$ 值<0.05 以及变化倍数>2 或<-2，方式一得到的 8 人差异蛋白中共有蛋白 25 个，方式二得到的 8 人差异蛋白中共有蛋白 35 个，这两种方式下共有蛋白交集 14 个，其中方式

一中有 5 个共有蛋白属于嗜中性粒细胞或白细胞的相关免疫通路，方式二中有 13 个共有蛋白属于白细胞介导的免疫，分别根据 25 和 35 个蛋白的基因功能富集到的生物学通路如下图。共有差异蛋白中和免疫相关的蛋白鉴定深度如下图。每名志愿者不同时间点差异蛋白数量及蛋白交集情况，如附图。

| 样本号 | 性别 | 年龄 | 收集到尿样数 | 鉴定蛋白质数（unique peptide >1） | 差异蛋白数（方式一） | 差异蛋白数（方式二） | 共有差异蛋白数 |
|---|---|---|---|---|---|---|---|
| 1 | 女 | 22 | 5 | 1557 | 503 | 540 | 399 |
| 2 | 女 | 22 | 6 | 1539 | 919 | 960 | 829 |
| 3 | 女 | 26 | 5 | 1652 | 855 | 981 | 774 |
| 4 | 男 | 24 | 6 | 1295 | 498 | 555 | 423 |
| 5 | 男 | 29 | 6 | 1346 | 756 | 784 | 653 |
| 6 | 男 | 24 | 6 | 1344 | 757 | 696 | 622 |
| 7 | 女 | 21 | 5 | 1775 | 782 | 744 | 581 |
| 8 | 男 | 24 | 6 | 1320 | 589 | 574 | 472 |




**8 名志愿者接种流感疫苗后尿液蛋白质反映的免疫相关通路不同**

我们对 8 名志愿者进行自身前后对照分析，采用如上所述两种方式得到差异蛋白，将每两个时间点得到的差异蛋白进行生物学通路分析。大部分人在接种后 24h 的尿样中富集到了和免疫相关的通路，表明疫苗开始发挥作用，人体开始产生免疫，不过具体通路不尽相同。我们分析可能因为 8 人中有些人在接种之前可能在生活环境中接触过四价流感中的病毒成分，接种疫苗后相当于二次免疫，同时也可能每个人体质不同，造成面对同样流感疫苗机体所产生的免疫反应也不同。3 号志愿者接种后 24h 未留尿液，只能采用第三个时间点和第一个时

间点比较。下表，列出了 8 人每两个时间点差异蛋白富集到的免疫相关通路（FDR < 0.05 且 *P*-vlaue < 0.05,浅蓝色标识的通路 *P*-vlaue < 0.05 但 FDR < 0.5，深蓝色标识的通路 *P*-vlaue < 0.05 但 FDR < 1）。这里只列举了免疫相关通路，该通路 *P*-vlaue、FDR 等相关参数以及差异蛋白富集到的其他生物学通路等信息见附表。

| | | |
|---|---|---|
| | **1 - 2** | |
| | innate immune response in mucosa | |
| | immune response | |
| | **1 - 3** | **2 - 3** |
| | defense response to Gram-negative bacterium | establishment of skin barrier |
| | inflammatory response | immune response |
| | **1 - 5** | **3 - 5** |
| **volunteer1** | receptor-mediated endocytosis | negative regulation of inflammatory response |
| | acute-phase response | acute-phase response |
| | | receptor-mediated endocytosis |
| | | innate immune response in mucosa |
| | | regulation of macroautophagy |
| | **1 - 6** | **5 - 6** |
| | none | defense response to Gram-negative bacterium |
| | | regulation of macroautophagy |
| | **1 - 2** | |
| | leukocyte migration | |
| | receptor-mediated endocytosis | |
| | acute-phase response | |
| | antibacterial humoral response | |
| | viral entry into host cell | |
| | innate immune response | |
| | inflammatory response | |
| | **1 - 3** | **2 - 3** |
| **volunteer2** | innate immune response | inflammatory response |
| | receptor-mediated endocytosis | innate immune response |
| | acute-phase response | positive regulation of viral release from host cell |
| | viral transcription | viral budding via host ESCRT complex |
| | defense response to Gram-negative bacterium | leukocyte cell-cell adhesion |
| | viral entry into host cell | positive regulation of T cell proliferation |
| | inflammatory response | negative regulation of T cell receptor signaling pathway |
| | antibacterial humoral response | viral entry into host cell |
| | viral life cycle | defense response to Gram-positive bacterium |
| | | regulation of immune response |

| | | |
|---|---|---|
| | | viral release from host cell |
| | | acute-phase response |
| | | viral life cycle |
| | **1 - 4** | **3 - 4** |
| | innate immune response | leukocyte migration |
| | acute-phase response | inflammatory response |
| | adaptive immune response | acute-phase response |
| | inflammatory response | viral release from host cell |
| | | immune response |
| | | viral entry into host cell |
| | | leukocyte cell-cell adhesion |
| | | innate immune response |
| | | defense response to fungus |
| | | regulation of immune response |
| | **1 - 5** | **4 - 5** |
| | viral transcription | innate immune response |
| | receptor-mediated endocytosis | acute-phase response |
| | | inflammatory response |
| | | viral entry into host cell |
| | | leukocyte migration |
| | **1 - 6** | **5 - 6** |
| | viral transcription | acute-phase response |
| | defense response to fungus | inflammatory response |
| | innate immune response in mucosa | |
| | **1 - 3** | |
| | viral transcription | |
| | **1 - 4** | **3 - 4** |
| | leukocyte migration | viral transcription |
| | viral entry into host cell | leukocyte migration |
| | innate immune response | viral entry into host cell |
| | regulation of immune system process | positive regulation of T cell proliferation |
| | acute-phase response | leukocyte cell-cell adhesion |
| **volunteer3** | antibacterial humoral response | |
| | **1 - 5** | **4 - 5** |
| | defense response to fungus | acute-phase response |
| | defense response to bacterium | leukocyte migration |
| | | innate immune response |
| | **1 - 6** | **5 - 6** |
| | viral transcription | viral transcription |
| | | receptor-mediated endocytosis |
| | | acute-phase response |
| **volunteer4** | **1 - 2** | |

| | | |
|---|---|---|
| | antifungal humoral response | |
| | **1 - 3** | **2 - 3** |
| | response to drug | antibacterial humoral response |
| | antibacterial humoral response | innate immune response |
| | | response to drug |
| | | regulation of inflammatory response |
| | **1 - 4** | **3 - 4** |
| | immune response | inflammatory response |
| | antibacterial humoral response | response to drug |
| | inflammatory response | immune response |
| | neutrophil migration | antibacterial humoral response |
| | | negative regulation of T cell apoptotic process |
| | | innate immune response |
| | **1 - 5** | **4 - 5** |
| | acute-phase response | viral release from host cell |
| | immune response | inflammatory response |
| | regulation of immune system process | |
| | inflammatory response | |
| | **1 - 6** | **5 - 6** |
| | leukocyte migration | viral entry into host cell |
| | viral entry into host cell | leukocyte migration |
| | acute-phase response | inflammatory response |
| **volunteer5** | **1 - 2** | |
| | leukocyte migration | |
| | acute-phase response | |
| | regulation of immune system process | |
| | innate immune response | |
| | **1 - 3** | **2 - 3** |
| | leukocyte migration | regulation of immune response |
| | viral entry into host cell | viral release from host cell |
| | innate immune response | leukocyte migration |
| | acute-phase response | phagosome acidification |
| | | viral life cycle |
| | | viral entry into host cell |
| | **1 - 4** | **3 - 4** |
| | leukocyte migration | receptor-mediated endocytosis |
| | receptor-mediated endocytosis | virion assembly |
| | acute-phase response | response to drug |
| | chaperone-mediated autophagy | |
| | innate immune response | |
| | **1 - 5** | **4 - 5** |
| | leukocyte migration | leukocyte migration |

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|---|---|---|
| | acute-phase response<br>positive regulation of T cell proliferation<br>leukocyte cell-cell adhesion | receptor-mediated endocytosis<br>acute-phase response<br>viral entry into host cell |
| | **1 - 6** | **5 - 6** |
| | leukocyte migration<br>acute-phase response<br>receptor-mediated endocytosis<br>positive regulation of T cell proliferation | negative regulation of viral process<br>antifungal humoral response |
| **volunteer6** | **1 - 2** | |
| | leukocyte migration<br>viral entry into host cell<br>defense response<br>acute-phase response<br>defense response to Gram-negative bacterium<br>regulation of immune system process<br>inflammatory response<br>response to drug<br>antibacterial humoral response<br>innate immune response | |
| | **1 - 3** | **2 - 3** |
| | leukocyte migration<br>defense response to Gram-positive bacterium<br>innate immune response<br>antibacterial humoral response<br>viral entry into host cell<br>regulation of immune system process<br>positive regulation of receptor-mediated endocytosis | immune response<br>antibacterial humoral response<br>inflammatory response<br>receptor-mediated endocytosis<br>acute-phase response<br>leukocyte migration<br>defense response to Gram-positive bacterium<br>positive regulation of receptor-mediated endocytosis |
| | **1 - 4** | **3 - 4** |
| | leukocyte migration<br>viral entry into host cell<br>innate immune response<br>inflammatory response<br>defense response to Gram-positive bacterium<br>regulation of immune system process<br>regulation of immune response<br>defense response to Gram-negative bacterium<br>receptor-mediated endocytosis<br>adaptive immune response | inflammatory response<br>response to drug<br>defense response to Gram-negative bacterium<br>negative regulation of T cell apoptotic process |
| | **1 - 5** | **4 - 5** |
| | leukocyte migration<br>inflammatory response | positive regulation of phagocytosis<br>cellular response to drug |

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|---|---|---|
| | regulation of immune response | acute-phase response |
| | viral entry into host cell | innate immune response |
| | regulation of immune system process | viral entry into host cell |
| | defense response to Gram-positive bacterium | response to antibiotic |
| | innate immune response | receptor-mediated virion attachment to host cell |
| | | defense response to Gram-negative bacterium |
| | **1 - 6** | **5 - 6** |
| | leukocyte migration | receptor-mediated endocytosis |
| | regulation of immune response | cellular response to drug |
| | innate immune response | response to drug |
| | response to drug | autophagy |
| | inflammatory response | |
| | viral entry into host cell | |
| | defense response to Gram-positive bacterium | |
| | defense response | |
| | **1 - 2** | |
| | defense response to fungus | |
| | innate immune response | |
| | **1 - 3** | **2 - 3** |
| | leukocyte migration | defense response to Gram-negative bacterium |
| | innate immune response | response to drug |
| | defense response to fungus | leukocyte migration |
| | leukocyte migration involved in inflammatory response | positive regulation of phagocytosis |
| | positive regulation of phagocytosis | defense response to Gram-positive bacterium |
| | | immune response |
| | | innate immune response |
| | | antibacterial humoral response |
| **volunteer7** | **1 - 4** | **3 - 4** |
| | phagocytosis | viral life cycle |
| | viral entry into host cell | positive regulation of viral release from host cell |
| | negative regulation of inflammatory response | viral budding via host ESCRT complex |
| | virion assembly | autophagy |
| | innate immune response | response to drug |
| | | innate immune response |
| | | regulation of viral process |
| | | viral release from host cell |
| | **1 - 5** | **4 - 5** |
| | leukocyte migration | leukocyte migration |
| | innate immune response | innate immune response |
| | viral budding via host ESCRT complex | viral release from host cell |
| | defense response to Gram-negative bacterium | defense response to Gram-positive bacterium |

| | | |
|---|---|---|
| volunteer8 | inflammatory response | |
| | **1 - 2** | |
| | viral entry into host cell | |
| | receptor-mediated endocytosis | |
| | receptor-mediated virion attachment to host cell | |
| | acute-phase response | |
| | defense response to Gram-positive bacterium | |
| | **1 - 3** | **2 - 3** |
| | innate immune response | innate immune response |
| | receptor-mediated endocytosis | antibacterial humoral response |
| | viral entry into host cell | acute-phase response |
| | response to drug | inflammatory response |
| | acute-phase response | |
| | **1 - 4** | **3 - 4** |
| | viral entry into host cell | antibacterial humoral response |
| | innate immune response | negative regulation of T cell apoptotic process |
| | receptor-mediated endocytosis | |
| | antibacterial humoral response | |
| | **1 - 5** | **4 - 5** |
| | receptor-mediated virion attachment to host cell | antibacterial humoral response |
| | receptor-mediated endocytosis | leukocyte migration |
| | viral entry into host cell | negative regulation of natural killer cell differentiation involved in immune response |
| | acute-phase response | |
| | **1 - 6** | **5 - 6** |
| | leukocyte migration | leukocyte migration |
| | receptor-mediated endocytosis | response to drug |
| | viral entry into host cell | immune response |
| | inflammatory response | defense response to fungus |
| | innate immune respon | viral entry into host cell |
| | | defense response to Gram-positive bacterium |
| | | defense response to bacterium |
| | | negative regulation of T cell proliferation |



## 讨论

我们首次通过尿液蛋白质组角度来探索接种疫苗后人体免疫反应过程，我们发现即使是接种同一种疫苗，尿液蛋白质组中差异蛋白富集到的免疫相关通路不尽相同。有趣的是我们发现接种 24h 后大部分志愿者的尿液中均富集到和免疫相关通路的差异蛋白，而一般血液中进行相关抗体的检测一般在 2~4 周之后，此次接种的四价流感疫苗研发机构对其受试者的血清中抗体滴度以及阳转率采集自免后 28 天后，我们的探索或许为日后疫苗验证提供一个新的思路，相对较早的验证疫苗的药效。对于不同人接种同样流感疫苗后，各个时间点的

差异蛋白及反映的免疫相关通路不同的原因，我们猜测一是因为四价流感疫苗包含有四种病毒类型，不同人可能在之前的生活中接触到这四种病毒的情况不同，有的人可能或多或少接触过，导致此次接种后相当于触发了二次免疫。或者我们猜测也可能是不同人的体质不同，所以接触到同种病毒后引发免疫的程度不同。总之，我们通过此次实验，发现：尿液蛋白质中可以较早的揭示免后人体的免疫应答，为疫苗药效验证提供新思路，同时发现不同人接种同一种流感疫苗后触发的免疫应答机制不尽相同。

